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BDRL, D/A ltr, 28 Sep 1971

TECHNICAL MANUSCRIPT 205

CHEMICAL AND PHYSICAL VARIABLES AFFECTING FLUORESCEIN ISOTHIOCYANATE AND ITS PROTEIN CONJUGATES

459385

FEBRUARY 1965

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

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CHEMICAL AND PHYSICAL VARIABLES AFFECTING FLUORESCEIN ISOTHIOCYANATE AND ITS PROTEIN CONJUGATES

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Project 1C622401A071

February 1965

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ABSTRACT

The effect of pH, time, temperature, and buffer system on the fluorescence, absorbance, and stability of fluorescein isothiocyanate and its protein conjugates was studied. The effect of these parameters on dye-binding procedures was also studied. The fluorescence of the isothiocyanate and of the protein conjugate was maximal at about pH 8.7 and pH 10.7, respectively. The stability of their fluorescence above pH 7 was affected adversely by increases in pH or temperature. The protein conjugate, however, showed maximum stability at pH 10.5 and above. This was confirmed by paper electrophoresis. The type of buffer [carbonate, phosphate, borate, tris(hydroxymethyl)aminomethane, and barbiturate] had little effect on fluorescence, but increased molarity of buffer reduced the fluorescence stability of the isothiocyanate. The absorbance of fluorescein isothiocyanate and its conjugates increased with increasing pH. In 1 N NaOH, the effect of pH variation on absorbance was minimized. By increasing the pH and temperature in dye-binding procedures, the desired degree of labeling can be obtained with short conjugation periods.

I. INTRODUCTION

The technique of fluorescein labeling of antibodies for the detection of antigenic material, histochemical staining, and quantitative antigen-antibody reactions has been used increasingly since Coons et al.[1] introduced fluorescein isocyanate as a labeling agent. Riggs[2] later substituted the more easily prepared and relatively more stable fluorescein isothiocyanate (FITC). Marshall et al.[3] eliminated the denaturing effect of organic solvents by adding the powdered FITC directly to the protein solution.

In determining the degree of labeling (fluorescein/protein ratios), absorbance measurements have been generally based on impure reference standards or on such substitutes as sodium fluorescein and aminofluorescein. Computations of dye content from such data have led to confusing and sometimes contradictory reports that defied comparative evaluations.

An important variable in the measurement of absorbance and fluorescence is the pH of the medium. Emmart[4] found that the absorbance of fluorescein increased with pH in the range of 2 to 8 and Nairn[5] reported that both absorbance and fluorescence of FITC-protein conjugates increased with pH in the range of 6 to 8. It was also noted in immunofluorescence of bacterial cells that the fluorescence was higher at pH 9 than at pH 7.2.[6] Nairn,[5] Tokumaru,[7] and Goldstein et al.[8] have reported on the effect of conjugation time (at 5 C) on the yield of labeled antibody. The effect of a third variable, temperature, has not been adequately investigated. The effects of these parameters on the fluorescence, absorbance, and stability of the fluorescein labeling agent and its protein conjugates as well as on the dye-binding procedures were in need of more intensive study. This investigation was undertaken in an effort to coordinate these and other variables with the purpose of resolving some of the confusing chemical and physical aspects of fluorescent antibody procedures.

Fluorescein isothiocyanate; rather than the isocyanate, was chosen for this study because of its availability as a chromatographically pure crystalline product.[9]

II. MATERIALS AND METHODS

A. BOVINE GAMMA-GLOBULIN (FRACTION II)

The powdered protein, obtained from Nutritional Biochemical Corporation, Lot #2009, was dissolved in 0.85% NaCl to prepare a solution containing approximately 28 mg of protein per ml.

B. BOVINE ANTI-*BRUCELLA ABORTUS* GLOBULIN

Antiserum was obtained from a cow infected with *B. abortus*. The serum was fractionated at pH 7.8 by the cold methanol precipitation method of Dubert.[10] The dialyzed globulin solutions were prepared for conjugation by diluting with 0.85% NaCl to obtain a final concentration of approximately 28 mg of protein per ml.

C. FLUORESCEIN ISOTHIOCYANATE (FITC)

Chromatographically and chemically pure FITC, isomer 1, was obtained from the Baltimore Biological Laboratory, Inc., Lot #209614.

D. ANTIBODY ACTIVITY AND STAINING PROCEDURES

Agglutination titers for *B. abortus* were determined by the standard macroscopic test-tube agglutination test[11] and staining intensity was determined by the fluorescent antibody reaction at pH 9.[6]

E. ESTIMATION OF PROTEIN AND FITC CONTENT

The biuret method of Gornall et al.[12] was previously described.[13] Protein absorbance was measured at 560 mμ,[14] where absorbance due to bound FITC is negligible.

Samples of FITC and its protein conjugates were taken up in 1 N NaOH and the absorbancies were measured at 490 mμ in a Beckman DU spectrophotometer. The absorbance of a solution, containing 2.0 μg FITC per ml of 1 N NaOH, was found to be 0.45.

The calculated molar ratios were based on molecular weights of 389 and 160,000 for FITC and globulins, respectively.

Absorption spectra were obtained on a Beckman DK-2A recording spectrophotometer.

F. PAPER ELECTROPHORESIS

Electrophoresis was carried out in a horizontal migration chamber as previously described.[13]

G. ESTIMATION OF FLUORESCENCE

The fluorescence of FITC and its conjugates was measured in a Beckman DK-2A spectrophotometer equipped with a spectral fluorescent attachment. The source of activating light was a low-intensity mercury lamp used in conjunction with a blue primary filter, Corning 5-61. No secondary filter was necessary. Fluorescence was recorded at the wavelength of maximum emission (520-521 mμ).

A 0.4- or 0.5-mm slit width and 0 to 10 per cent transmission scale were used. The concentration curves of FITC and its protein conjugates followed Beer's law of linearity from about 0.1 to 2.0 μg per ml and 0.3 to 5.0 μg per ml, respectively. Fluorescence emission was standardized with an artificial fluorescent reference supplied with the fluorescent attachment.

H. CONJUGATION

The conjugation procedure of Marshall et al.[3] was modified by dissolving the FITC in 0.5 M carbonate buffer of a given pH prior to its addition to the protein sample. The freshly prepared FITC solution was added immediately to a 2.8 per cent protein solution in the ratio of 1:10. The FITC-protein mixture contained 40 μg FITC per mg of protein. Conjugation was allowed to proceed at room temperature (23 to 26 C) or at 5 C with continuous mixing over a magnetic stirrer. The sample was equilibrated to temperature before addition of the FITC solution and the pH of the conjugate mixture was measured during conjugation. Unreacted FITC was removed by passing the conjugate mixture through a Sephadex column (G-25, medium grade) as described by Gordon,[16] using 0.85% NaCl for equilibration and elution at room temperature. The pH of the eluate from the Sephadex column was usually between 7.2 and 7.7.

When FITC is dissolved in the 0.5 M carbonate buffer, the pH drops as the acidic FITC neutralizes alkali. For example, treatment of the 2.8 per cent globulin solution with 40 μg of FITC per mg protein in carbonate buffer at pH 9.0 results in a drop of about 0.3 pH unit. The lower pH is maintained for at least an hour at room temperature and for about 3 hours at 5 C. At the latter temperature, the pH appears to rise after 18 hours to about pH 9.

I. PREPARATION OF SAMPLES FOR FLUORESCENCE AND ABSORPTION STUDIES

A stock solution of FITC was prepared in acetone. This was diluted 1:70 with appropriate buffer solutions. A similarly prepared acetone blank showed negligible fluorescence or absorbance. Samples of a freshly prepared FITC-protein conjugate, free of unreacted FITC, were diluted 1:70 with appropriate buffer solution and the initial fluorescence was measured immediately. The absorbance at 490 mμ and the pH of each solution were also recorded. The solutions were then allowed to stand at room temperature or at 5 C and the fluorescence was measured after various time intervals. Each set of data, totalling 26 to 40 samples, was accumulated from a series of experiments. No effort was made to maintain a constant sample pH from one experiment to another. The curves, fitted to the plotted data, were drawn to show a trend rather than absolute transmission values.

A 0.01 M phosphate buffer was used in the range of pH 7.0 to 8.5 and a 0.01 M carbonate buffer between pH 9.0 and 11.0. For higher pH levels, the samples were taken up in 0.01 M and 0.1 M NaOH solutions.

III. RESULTS

A. EFFECT OF pH ON FLUORESCENT EMISSION AND ABSORBANCE OF FITC AND ITS PROTEIN CONJUGATE

Figure 1 shows the fluorescence at zero time and absorbance of FITC solutions as a function of pH. The fluorescence intensity increases to a peak at about pH 8.7 and then rapidly decreases. The fluorescence at pH 8.7 is about 10 per cent greater than at pH 7.0. In a 0.1 N NaOH solution (not shown), there is a fourfold drop from maximum fluorescence at pH 8.7; in a 1 N NaOH solution (not shown), the decrease is almost sixfold. When solutions of FITC above pH 8.7 are immediately titrated back to this pH there is no increase in fluorescence, indicating that the pH effect on fluorescence is not reversible.

The absorbance progressively increases with pH up to about pH 13 (0.1 N NaOH). No further changes occur beyond this pH. The absorbance of an FITC solution in a 0.1 N NaOH or 1 N NaOH is about 1.5 times greater than at pH 7.0.

The effect of pH on FITC-protein conjugates is shown in Figure 2. The fluorescence increases with pH to approximately pH 10.7 at which point the intensity is almost twice that at pH 7.0. Beyond pH 12, the fluorescence drops sharply, so that the intensity in a 1 N NaOH solution (not shown) is only 1/18 that found at pH 10.7. When the conjugate in the 1 N NaOH is readjusted to pH 8.3, the fluorescence becomes significantly greater and is about equal to that of the same conjugate taken up directly in a buffer solution of pH 8.3. Additional studies in the alkaline range confirm the reversibility of conjugate fluorescence with change in pH.

The absorbance curve of the FITC-protein conjugate shows a progressive increase similar to that in the FITC absorbance curve except that a slight peak appears in the 1 N NaOH. The absorbance, at its peak, is about 1.5 times greater than at pH 7.0.

The loss of fluorescence intensity upon binding FITC to protein was calculated from the absorbance and fluorescence data for FITC and its conjugates. This loss decreases with increasing pH, being about 30 per cent higher at pH 7.0 than at pH 11.0. Goldman and Carver[16] found a 90 per cent drop in fluorescence intensity and McDevitt, et al.[17] found an 82 per cent drop. This discrepancy may be caused at least partly, by differences of pH during measurement.

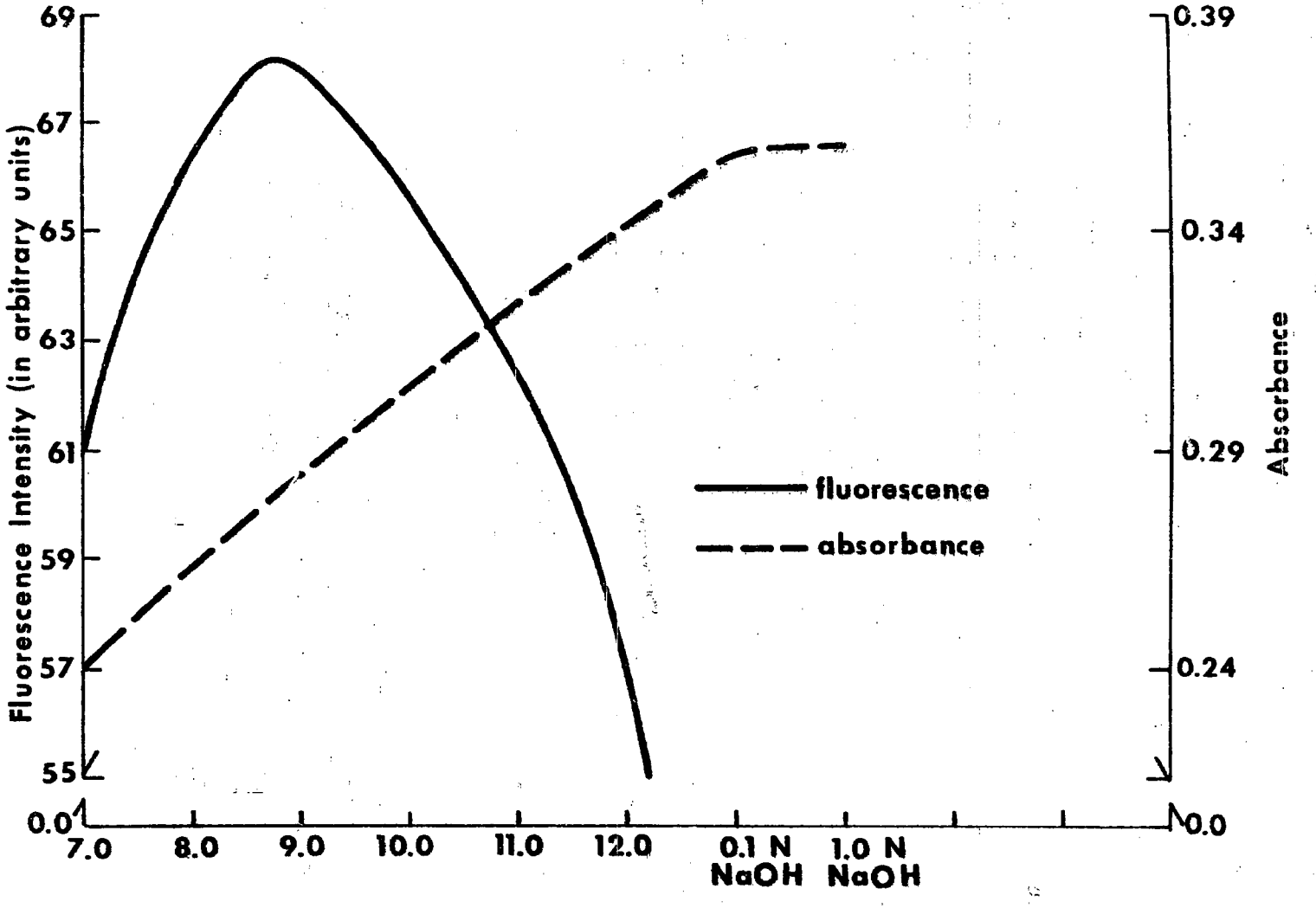

Figure 1. Fluorescence Emission and Absorbance of FITC Solutions as a Function of pH.

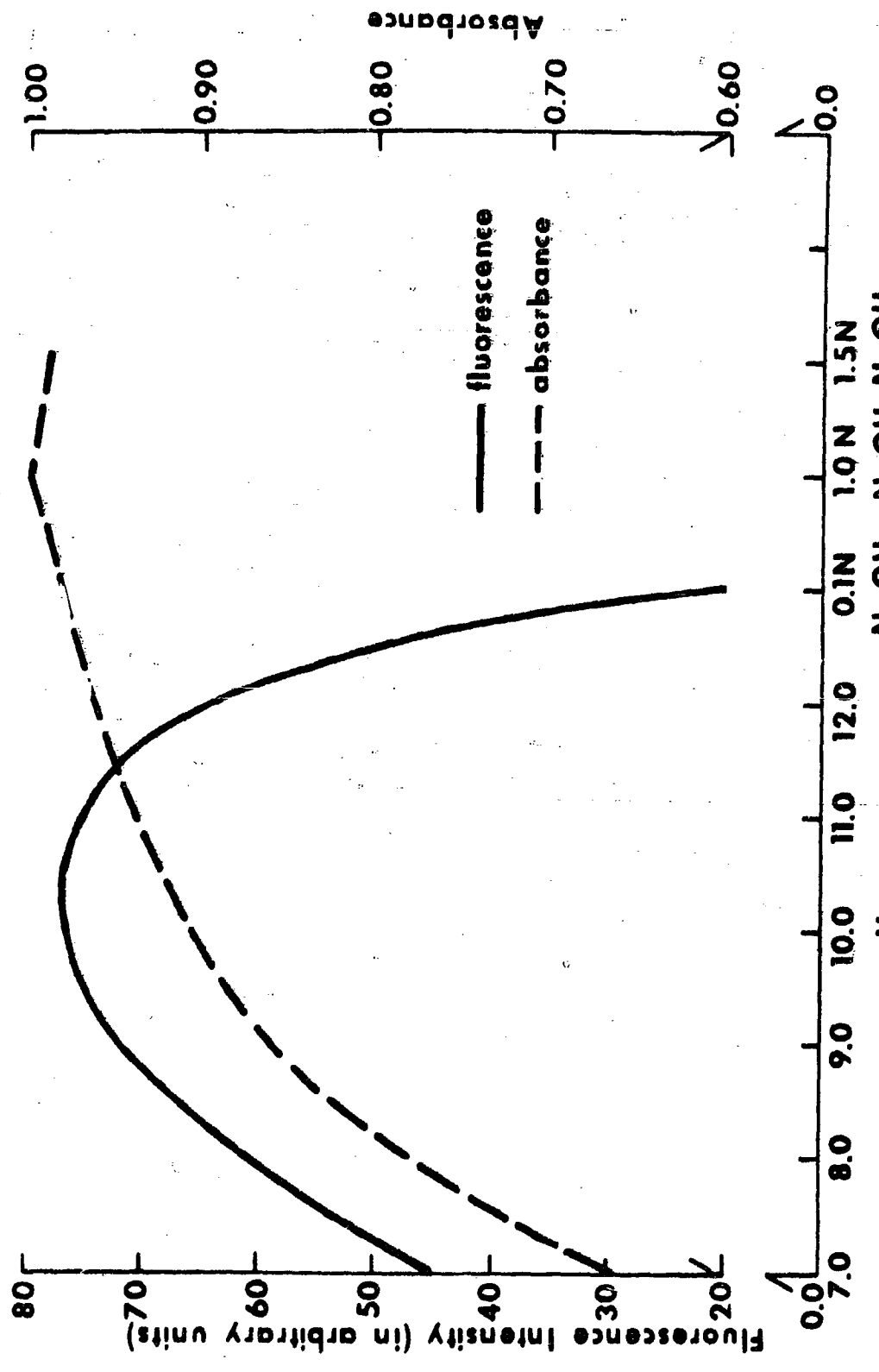

Figure 4. Fluorescence Emission and Absorbance of FITC-Protein Conjugates as a Function of pH.

8. EFFECT OF pH AND TEMPERATURE ON STABILITY OF FLUORESCENCE

Figure 3 shows the effect of time on the fluorescence intensity of FITC solution at room temperature. The zero time curve was reproduced from Figure 1 for comparison. The flattened appearance of this curve is the result of extending the ordinate scale. After 4 hours, small losses of fluorescence occur in the pH range of 7-9; there are somewhat larger losses above pH 9. After 24 and 72 hours, losses are observed at all pH levels, the amount of loss increasing with time. At 5 C, a similar drop in fluorescence occurs at the various pH levels but less rapidly. At pH 8.7, for example, the loss after 72 hours is only about 19% compared with 60% at room temperature. In a 1 N NaOH solution (not shown), FITC is converted rapidly to more stable, less fluorescent substances because little further change in fluorescence occurs with time.

The effect of time on the fluorescent intensity of protein conjugates as a function of pH is shown in Figure 4. The zero time curve was reproduced from Figure 2 for comparison. There is essentially no loss of fluorescence after 4 hours at room temperature. After 24 hours, slight losses occur beyond pH 9.6. Thereafter, the fluorescent behavior of the conjugates is unlike that found for FITC solutions. In the pH range of 7 to 10, a general increase in fluorescence occurs that continues to rise for about 4 to 6 days and then slowly decreases. The conjugate appears to demonstrate minimum stability of fluorescence at about pH 8.8 and maximum stability at pH 10.5 and above. At 5 C, similar changes take place at a much slower rate.

The effect of time on FITC solutions and its protein conjugates was also studied electrophoretically. The FITC solutions, at pH 7.8 and in a 1 N NaOH solution, were allowed to stand for several days at room temperature. At intervals during this period, samples were removed and subjected to electrophoresis. The same procedure was applied to freshly prepared conjugates (free of unreacted FITC), which were buffered at pH 7.7, 9.5, and 11.0. The FITC, at pH 7.8, produces a single yellow-colored band. In 1 N NaOH, the band of "degraded" FITC has an orange color and migrates at a slightly faster rate than unchanged FITC. After 2 to 3 days, the neutral FITC solution develops a second band that migrates at the same rate as "degraded" FITC. The alkaline FITC solution also deteriorates further to produce two additional bands. Although some of the bands are not visible under ordinary light, all bands show yellow fluorescence under ultraviolet light. The conjugate buffered at pH 7.7 and, to a lesser extent, that at pH 9.5 produce two protein-free fluorescent bands after 2 days. The minor band migrates at the same rate as the "degraded" FITC; the major band travels at a rate intermediate between the protein conjugate and the minor band. A fast-moving, third fluorescent band begins to develop after 8 days. The conjugate at pH 11.0 produces no protein-free fluorescent bands until the 10th day.

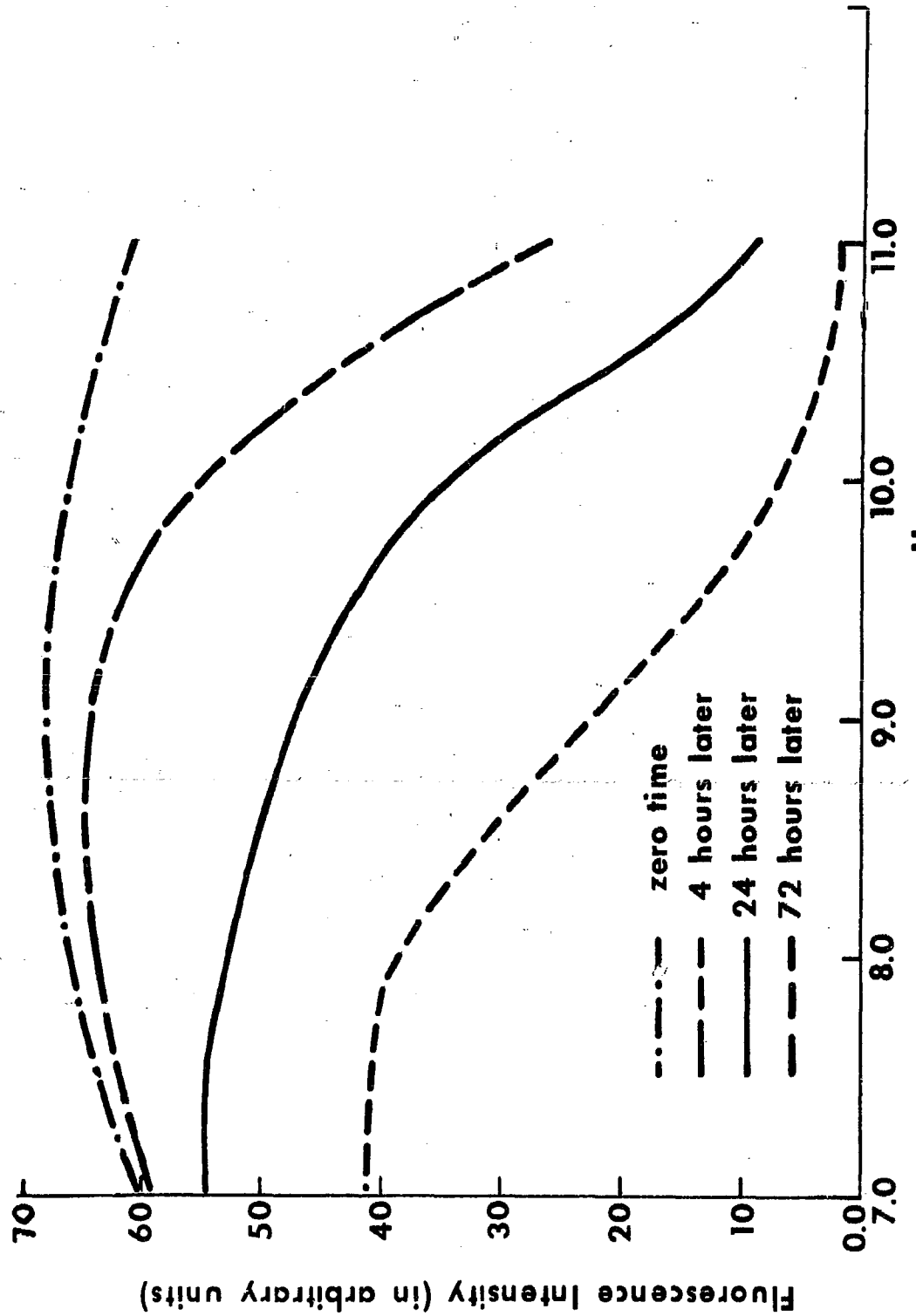

Figure 3. Loss of Fluorescence of FITC Solutions with Time as a Function of pH (at room temperature).

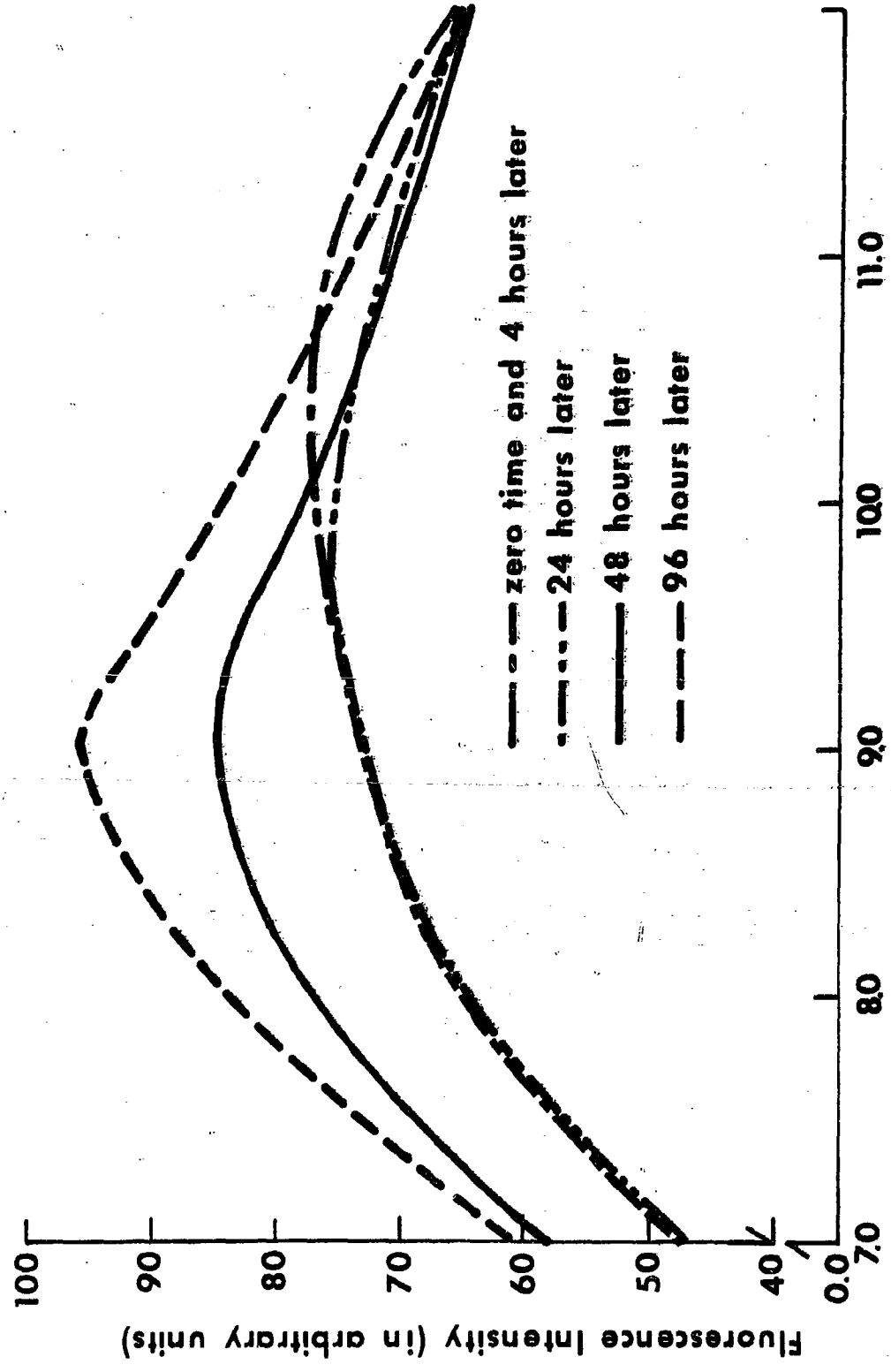

Figure 4. Loss of Fluorescence of FITC-Protein Conjugates with Time as a Function of pH (at room temperature).

C. EFFECT OF BUFFER SYSTEM AND CONCENTRATION ON FLUORESCENCE AND STABILITY OF FLUORESCENCE OF FITC AND ITS PROTEIN CONJUGATES

The samples were taken up in phosphate, carbonate, borate, tris(hydroxymethyl)aminomethane (tris), and barbiturate buffers at about pH 9 in concentrations ranging from 0.01 M to 1.0 M.

In general, the data showed that the type of buffer has a slight but insignificant effect on the initial fluorescence of FITC solutions or on stability of fluorescence. However, with increasing molarity sharp reductions in stability of fluorescence are observed, regardless of the buffer used. In a 0.05 M carbonate buffer, for example, there is about an 80% loss of fluorescence intensity at room temperature and a 35% loss at 5 C after 24 hours. This is more than twice that found in a 0.01 M carbonate buffer. With samples of FITC-protein conjugates, the data show that the type of buffer and its molarity seem to have little or no effect on the degree or stability of fluorescence.

D. FACTORS AFFECTING CONJUGATION YIELDS

The effect of pH, temperature, and conjugation time on the yield of labeled bovine γ-globulin and bovine anti-brucella globulins is shown in Table 1. The two types of globulins show only slight differences in reactivity toward FITC. Conjugation at pH 9.45, room temperature, for 30 minutes gives slightly greater yields than conjugating at pH 8.75, 5 C, for 18 hours. It will be noted that little further binding of the dye occurs as the yield approaches 10 to 12 moles FITC per mole protein (or about 65% of introduced dye), indicating apparent equilibrium with the protein-reactive groups. In a plot of conjugation yield vs. pH (Figure 5) we found that the yields after 15 minutes at room temperature closely parallel but are slightly greater than those at 5 C after 2 hours. The dye content of the conjugate prepared at pH 9.5, at either temperature, is approximately twice that at pH 8.7. From these data, it is evident that the degree of protein label varies with pH, temperature, and conjugation time.

The conjugated bovine anti-brucella globulin samples, shown in Table 1, and an unconjugated globulin sample were examined simultaneously for antibody activity by the tube agglutination test. All samples were diluted to contain 1 mg of protein per ml. With the use of twofold dilutions in 0.85% NaCl to a final dilution of 1:320, no apparent loss of activity as the result of conjugation was observed. The staining quality of the conjugates was evaluated by the microscopic fluorescent antibody reaction. Samples with molar ratios of 7 or above gave a 3+ to 4+ staining intensity. The sample with a molar ratio of 5.2 gave a 2+ to 3+ reaction.

TABLE 1. EFFECT OF pH, TEMPERATURE, AND TIME ON CONJUGATION YIELDS[a/]

Room Temperature			Temperature, 5 C		
pH of Conjugate Mixtures	Conjugation Time, minutes	F/P Molar Ratio	pH of Conjugate Mixtures	Conjugation Time, hours	F/P Molar Ratio
8.75 (B-G)	15	3.5	8.75 (B-G)	2	3.2
	60	9.2		3	4.3
				6	6.0
				18	10
9.45 (B-G)	15	8.2	9.25 (B-G)	0.5	2.5
	30	11		1	3.9
	60	12		2	5.7
				3	7.6
			8.75 (ABBG)	18	10
9.50 (ABBG)	15	7	9.50 (ABBG)	1	5.2
	30	9.5		2	7.5
	45	10		3	9.1

a. A 2.8 per cent bovine γ-globulin (B-G) or bovine anti-brucella globulin (ABBG) was conjugated with 40 μg FITC/mg protein. The unreacted FITC was removed with a Sephadex column. The F/P ratios of the protein conjugates are given in terms of moles FITC/mole protein.

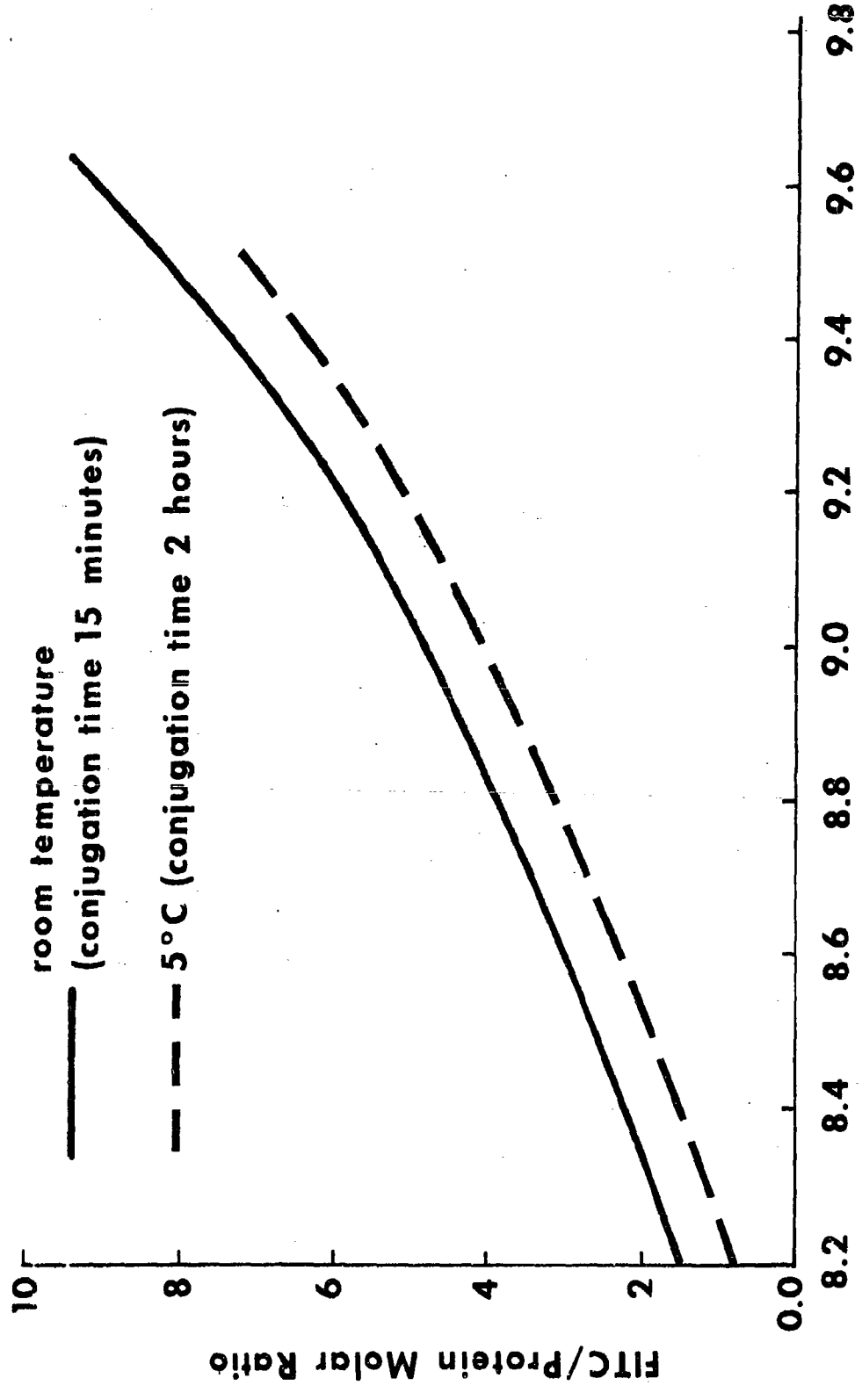

Figure 5. Conjugation Yield as a Function of pH.

The use of powdered FITC according to the method of Marshall et al.[3] resulted in lower yields with short conjugation times but only slight differences after an 18-hour conjugation. Borate or tris buffers could be substituted for the carbonate buffer in the conjugation reaction without significantly affecting the yield of conjugate, but the carbonate buffer showed greater buffering capacity above pH 9. The amino group in tris showed no evidence of interfering with the protein binding of FITC during conjugation.

IV. DISCUSSION

The fluorescence of fluorescein is believed to be due to enol-keto tautomerism involving the carboxyl group and one of the two phenolic groups.[18] In an acid solution, the carboxyl group forms a cyclic "lactoid" ring that prevents the tautomeric shift and thus inhibits fluorescence. This ring is disrupted in alkaline solutions, with the result that resonance is enhanced and fluorescence intensified. Fluorescence may be similarly affected by the type of substituent on the aromatic ring containing the carboxyl group[19] relative to its ability to attract or repel electrons.

In solution, FITC tends to hydrolyze to the less fluorescent aminofluorescein, which may, in turn, react with unchanged FITC to form the thiourea derivative.[20] The major band of "degraded" FITC, observed electrophoretically, is evidently aminofluorescein. The two bands that later develop probably consist of difluorescein thiourea and a product that is the result of other secondary reactions. Since the three bands show fluorescence, it is apparent that little or no alteration of the "fluorescein" fluorophor had taken place. Hydrogen-bonding or other solvent effects may account for the increase of FITC fluorescence to pH 8.7. The drop in fluorescence with further increases of pH is caused by degradation of FITC. In a 1 N NaOH solution, degradation appears to occur almost instantaneously.

The decrease in electron density of FITC probably accounts for the loss in fluorescence intensity as FITC binds to protein. The physical configuration of the protein may also be a factor in influencing the fluorescence of bound FITC. The exposure of previously masked conjugation sites to fluorescence activation by an unfolding of the protein molecule would explain the greater enhancement of conjugate fluorescence and its broader "fluorescence vs. pH" curve as compared with free FITC (Figures 1 and 2). The drop in conjugate fluorescence beyond pH 10.7 may be explained on the basis of ionization of the second phenolic group of the fluorescein moiety, which could conceivably prevent the tautomeric shift necessary for fluorescence or an augmented inductive effect as further ionization of the protein takes place. However, the apparent irreversibility of FITC fluorescence with change

of pH does not support the former view. Although the thiocarbamido bond of the FITC-protein conjugate shows great stability, some dissociation does occur with time. The liberated fluorescent substances, one of which is probably aminofluorescein, would account for the increase of conjugate fluorescence on standing. The conjugate appears to be most stable in the vicinity of maximal fluorescence, i.e., pH 10.7. The effect of deep-freeze storage at this high pH on antibody activity has not been studied.

The light-absorbing properties of FITC and its protein conjugate, unlike fluorescence, are not adversely affected in strongly alkaline solutions. FITC and its conjugate show an increase of absorbance with pH up to about pH 13 (0.1 N NaOH). In this pH range, the absorption maxima of FITC (490 mμ) and its conjugate (493 mμ) differ slightly. The peak absorbance of the conjugate observed in 1 N NaOH is caused by a shift of maximum absorption to 490 mμ. Absorbance measurements of FITC and its conjugate represent the over-all effect of absorption and fluorescence emission, expecially in the vicinity of 490 mμ, the wavelength of greatest excitation. As already noted, the effect of fluorescence is minimized in highly alkaline solutions. Consequently, the use of 1 N NaOH for absorbance measurements should reduce the effect of pH variation and insure maximum sensitivity.

The conjugation reaction may be described as an electrophilic attack of a positively charged isothiocyanate group on the protein at the amino group of highest electron availability. Generally, the protein is labeled at 5 C for 18 hours, using a 0.5 M carbonate buffer, pH 9.0. Frommhagen and Spendlove[21] recommended a buffer with a pH not exceeding 8.8 as a means of reducing the breakdown of FITC. However, this tends to lower the charge differential between the isothiocyanate grouping and the amino groups of the protein and, consequently, the yield of bound dye. Others[7,22] have used the pH range from 9.0 to 9.5 without any apparent justification for doing so. The usefulness of the higher pH values, however, is borne out by data reported in this paper.

The degree of labeling is also dependent on time and temperature, the amount of dye introduced into the sample[7,22] and the type of protein under study.[21] The rapid deterioration of FITC in 0.05 M carbonate buffer, even at 5 C, reduces the labeling efficiency during long conjugation periods. The use of freshly prepared FITC solutions in buffer permits the use of short conjugation times at increased temperatures. Low F/P ratios have been recommended for greater specificity in histochemical and viral staining, but when essentially no contaminating host material is present, as with "clean" bacterial suspensions, high F/P ratios seem to afford the greatest intensity of staining.[23] Bovine anti-brucella globulins with F/P ratios of 7 and above were found to have good staining qualities.

It is believed that commonly used procedures for fluorescent antibody studies should be re-evaluated, particularly on the basis of pH. In the preparation of protein conjugates, the desired degree of labeling may be obtained rapidly by a judicious variation of pH and temperature.

V. SUMMARY

The effect of pH, time, temperature, and buffer systems on solutions of fluorescein isothiocyanate and its protein conjugates was examined fluorometrically. The fluorescence of the isothiocyanate and of the protein conjugate was maximal at about pH 8.7 and pH 10.7, respectively. The stability of their fluorescence above pH 7 was affected adversely by increases in pH or temperature. The protein conjugates, however, showed maximum stability at pH 10.5 and above. The type of buffer (carbonate, phosphate, borate, tris, and barbiturate) did not affect the fluorescence of the free dye significantly. On the other hand, increasing the molarity of the buffer caused a decrease in stability of fluorescence of the free dye but did not seriously affect the fluorescence of the conjugate.

The absorbance of fluorescein isothiocyanate and its conjugates increased with increasing pH. In 1 N NaOH, the effect of pH variation on absorbance was minimized.

By increasing the pH or temperature of the reaction mixture during conjugation, fluorescein isothiocyanate reacted more readily with the protein. Conditions may be selected to obtain the desired degree of label with short conjugation periods. Conjugation of a bovine anti-*Brucella abortus* globulin sample for 30 minutes at pH 9.45 and room temperature was as effective as conjugating at pH 8.75 for 18 hours at 5 C. No apparent loss of biological activity as the result of conjugation was observed.

The binding of fluorescein isothiocyanate to protein resulted in a loss of fluorescence efficiency, the extent of loss being dependent upon the pH of measurement.

Existing procedures in fluorescent antibody studies should be re-evaluated on the basis of the variables discussed.

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